LC/MS

A Practical User's Guide

MARVIN C. McMASTER

A JOHN WILEY & SONS, INC., PUBLICATION

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To the memory of my son, Chris McMaster, my writing partner and the artist on the first two books in this series. Chris has passed on to bigger and better things painting sunrises and rainbows.

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PREFACE

I consult and teach extension courses on laboratory instrumentation and computers at the University of Missouri–St. Louis. I taught a course called *Practical HPLC* for a number of years while working as a sales representative and technical support specialist for a variety of instrument companies. The first book in this series, *HPLC: A Practical User's Guide*, arose out of a need for a textbook for my course. At the end of that book I wrote a chapter on a rising research technique that I felt would eventually transform the life of the average laboratory chemist and provide a tool for definitive identification of the compounds that he or she was producing.

I next had an opportunity to work with a manufacturer of control and data systems for GC/MS equipment. I added consulting and teaching in this specialty to my portfolio and designed a book, *GC/MS: A Practical User's Guide*, to provide a teaching tool. Again, I added a final chapter on the growing art of LC/MS. I feel another book and course are needed now that commercial sales of LC/MS systems has nearly equaled those of GC/MS systems. This tool combines my expertise and interests in several separations areas.

I do not attempt to write the definitive book for a new instrumentation specialty. I want to put together a useful tool for introducing the technique and providing practical information on how to use it. I try to look at complicated material, internalize it, and present it in a way that is understandable and useful for solving laboratory problems. When inexpensive, easy-to-use LC/MS systems appear on the end of every laboratory bench, I would like to have a copy of this book setting next to them to lay the groundwork for getting the most out of the system.

When I teach practical courses, I use an overhead projector and a PowerPoint slide set to provide the theme and illustrations for the course. I realize that

if I were buying this book to use as a teaching text book, it would be very useful to have the slide set on a CD/ROM disk. In the back of this book I have included such a disk with my slide set, searchable files on LC/MS Frequently Asked Questions, a glossary of terms, and useful LC/MS tables. For the LC/MS students, this provides a series of self-study guides for learning or honing their LC/MS skills. I hope the readers of this book will find these additional tools useful. I plan to add similar tools to later editions of my other books.

I wish to thank the following companies for permission to use drawings and illustrations from their brochures and Web sites: Agilent Technologies, Applied Biosystems, ESA, Varian, and Waters Corporation. I have found in teaching that pictures truly are worth a thousand words. Their kind assistance has helped me keep this book down to a reasonable size. I never have cared for "rat killer" manuals.

MARVIN C. MCMASTER

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INTRODUCTION TO LC/MS

Liquid chromatography (LC) combined with mass spectrometry (MS) creates an ideal analytical tool for the laboratory. The high-performance liquid chromatograph (HPLC) has been the laboratory tool of choice for separating, analyzing, and purifying mixtures of organic compounds since the 1970s.

An HPLC column can separate almost any mixture that can be dissolved. A mass spectrometer can ionize the separated peak solution and provide a molecular weight for each peak component. An LC/MS/MS system can fragment the parent ion into a distinctive fragmentation pattern and can separate the daughter ions for identification and quantitation. The characteristic fragmentation pattern from each parent ion can be identified by comparison to fragmentation patterns produced by standard computerized databases. The output of the HPLC system can be divided for analysis by other HPLC detectors or for preparative sample recovery, since only a small portion of the column effluent is required for mass spectral analysis.

1.1 WHY LC/MS?

The preferred tool until the turn of the millennium for separating a mixture and providing definitive identification of its components was the gas chromatograph/mass spectrometer (GC/MS). However, this technique was limited by three main factors:

1. Sample volatility.
2. The fact that aqueous samples require extraction.
3. Thermal degradation of samples in the GC oven.

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Not all compounds are volatile enough to be introduced or eluted off a GC column. Aqueous mixtures have to be extracted and/or derivatized before injection, adding to analysis cost and bringing sample handling errors into peak quantitation. The columns available were not able to resolve all mixtures of compounds. This problem has been eliminated somewhat with new varieties of columns. Oven-temperature programming remains the principal variable available for separating compounds in a mixture. The final oven temperature necessary to remove a large compound from a column can degrade many thermally labile compounds.

In the last two years, LC/MS sales have nearly equaled GC/MS sales because of the additional compounds that can be analyzed by LC/MS and the greater range of separation variables that can be utilized in HPLC separation. The editors of *Analytical Instrumentation Industry Reports* say that in 2000 the global GC/MS market was $300 million and that LC/MS sales reached $250 million. This does not indicate parity, but it does show that the gap is closing. One industry analyst predicted that LC/MS sales should top $1 billion by 2005. The difference in cost of a HPLC system and its interface compared to a gas chromatograph must be factored into these numbers when comparing unit costs. An isocratic HPLC system costs 50% more than a basic GC module. The cost difference nearly doubles when you add in the cost of an atmospheric-pressure interface (API). Gradient HPLC configuration increases the cost to triple that of a GC module. However, all of these costs are overshadowed by the price of a mass spectrometer.

For LC/MS to be a major player in the analytical laboratory, there are factors limiting performance that must be overcome:

- Analyzer signal swamping by the elution solvent.
- Solvent composition changing in gradient elution.
- Buffer use for pH control.
- Ionization of neutral peak components.

By far the most important of these is the volume of eluting solvent necessary to displace the compounds separated from the HPLC column. The mass analyzer is quickly overwhelmed by the signal from the solvent if the HPLC output is introduced directly into the mass spectrometer. The analyte signal is buried beneath this solvent signal avalanche. The solvent signal saturation effect occurs even if a low-molecular-weight solvent such as methanol or water is chosen and a low analyzer mass cutoff range is selected to exclude the solvent's peak signal. A method for in-stream solvent removal with concurrent sample concentration must be provided to connect the column effluent to a high-vacuum mass spectrometer. The HPLC solvent gradient used to resolve closely eluting HPLC peaks and decrease HPLC run times also produces solvent composition changes that further complicate the solvent-masking effect of analyate signal.

Many compounds resolved by the HPLC column require pH control to adhere to the column long enough to be eluted. Removal of nonvolatile buffer and ion-pairing reagents commonly used in HPLC separations from the effluent is the next

problem that must be handled. Direct introduction of inorganic compounds into a high-vacuum system will cause mass spectrometer inlet fouling and loss of signal. Organic buffers used instead of inorganic buffers exhibit the same problems as those found with organic solvents: They overwhelm the analyzer and detector. Replacing nonvolatile buffers and reagents with volatile equivalents allows them to be removed like solvent. The final hurdle is that neutral compounds separating off the HPLC column must be converted to charged molecular ions or fragmented into charged ions that can be separated by the analyzer.

API using ion spray and electrospray interfaces provides many of the answers to these problems. At least part of the stream from the HPLC is sprayed over a high-voltage coronal discharge needle in a heated chamber, vaporizing the solvent and charging the suspended molecule, creating a molecular ion. A neutral flowing curtain gas sweeps much of the solvent and volatile additives out of the interface before the ionized analyate is pulled into the pinhole entrance to the high-vacuum environment of the analyzer. One of Jack Henion's papers produced at Cornell University reports that he operated an ion spray interface at effluent flow rates of 2 mL/min of methanol/water containing phosphate buffer to feed sample into a Hewlett-Packard MSD mass spectrometer with its vacuum provided by a tiny turbo pump, but this should be looked on as an exception to the rule of using volatile components.

Liquid chromatography provides a wide variety of operating variables that can be used to control and optimize a separation:

- Column-bonded phase selection with rapid column switching.
- Major solvent change with rapid reequilibration.
- Mobile-phase polarity adjustment and gradient operation.
- Packing support selection for pH and temperature stability.
- Temperature programming.

Most HPLC separations have been carried out using reverse-phase silica columns, with non-polar-bonded phases eluting compounds with polar solvents. A wide variety of bonded phases are available to achieve these separations. Various nonpolar mobile-phase solvents can be selected to shift elution orders of compounds on the same type of column. Mixing nonpolar solvents with water can change solvent polarity, increasing or decreasing partitioning with bonded-phase packing.

Traditional HPLC column supports have had nonpolar bonded phase bound to a silica matrix. These bonded phases are unstable under strongly acidic conditions, and the silica matrix dissolves rapidly at mildly basic pH. Newer polymeric and zirconium matrixes provide reverse-phase columns that are both pH and temperature stable. These packing materials allow operation at high or low pH without using buffers. Zirconium packing allows use of temperature as a separations variable using a temperature-controlled column jacket. Thermally labile compounds would have some of the same problems as those seen in a GC oven, but the temperature control range is much lower in HPLC, due to solvent volatility.

In the first section of this book we focus on optimization of the liquid chromatograph. We discuss equipment configurations, columns, and separation variables that can help improve peak resolution. Routine maintenance tips will show how to maintain the system and the separation without contaminating the interface and the mass spectrometer. An earlier book in this series, *HPLC: A Practical User's Guide*, provides additional information on using and optimizing the performance of silica-based HPLC columns.

In the second section of the book we look at the components that make up the various analyzers used in mass spectrometry. We compare the advantages and areas of specific applications of quadrupole, ion trap, Fourier transform, and time-of-flight (TOF) analyzer configurations. A variety of systems for generating the high vacuum used in analyzer operations are described and evaluated. Techniques for maintain a system under operating conditions and for cleaning contaminated analyzers are explained. The basic theory for controlling analyzer and detector sensitivity and scanning ranges is discussed. Two of the great advances in interpretation of mass spectral data have been the introduction of accurate mass-molecular-weight determination and computer scanning of library databases of known fragmentation patterns to aid compound identification. These have greatly reduced the time and operator skills needed to use and understand information generated by mass spectrometers. A brief introduction to fragmentation pattern interpretation from LC/MS/MS data is provided to aid in checking database search results.

1.2 MOLECULAR WEIGHTS AND STRUCTURE STUDIES

In the final section of the book we look at a number of current application areas for LC/MS in biochemical and industrial laboratories as well as other areas of LC/MS application that are anticipated when regulated methods become available. Special emphasis is placed on drug discovery and development, protein analysis, impurities, and metabolite determinations. These areas have fueled the rapid growth of LC/MS sales in the last few years. The needs of these labs go beyond the desire to provide separation and molecular weights for compounds in synthesis mixtures. Fragmentation studies using LC/MS, LC/MS/MS, and mixed analyzer systems to supplement LC/MS comprise a rapidly growing technology. It is important to understand the changes in system costs, hardware configurations, applications, and techniques that seem to be driving these changes.

1.3 LC/MS SYSTEMS

Basically, an LC/MS system is an HPLC pumping system, injector, and column married to a mass spectrometer through some type of evaporative ionizing interface (Figure 1.1). A computer system coordinates the components of the system together by providing control of the HPLC for flow, solvent gradient, and remote starting of injection and the gradient run. It also provides control of the mass